EVALUATION OF PURKINJE NEURONS AND ENDOCANNABINOID RECEPTORS AS A POTENTIAL TARGET OF ALCOHOLISM USING JAPANESE MEDAKA (*ORYZIAS LATIPES*) AS AN ANIMAL MODEL

by
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Abstract

Evaluation of Purkinje neurons and endocannabinoid receptors as a potential target of alcoholism using Japanese medaka (*Oryzias latipes*) as an animal model

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(Under the direction of Dr. Asok Dasmahapatra)

Purkinje cells (PK) are neurons with large cell bodies found in the central nervous system and are mostly distributed in the cerebellar region of the hindbrain. Several novel markers for PK cells have been developed and are used for characterization of neurobehavioral disorders. The present study was aimed to identify endocannabinoid receptor 1 (CB_1) as a potential marker of PK cells and evaluate them as a molecular target of alcohol and cerebellar functions using Japanese medaka (*Oryzias latipes*) as an animal model. Previously, we have observed that medaka genome consist three CB receptor paralogs; two of them (*cnr1*a and *cnr1b*) showed structural identity with human. We have also observed that expression of *cnr1a* mRNA in medaka embryos was disrupted by developmental ethanol exposure. Moreover, exposure to waterborne ethanol (300mM) is able to alter the swimming behavior of the adult male medaka within 1 hour of exposure. Preliminary data indicate that the CB_1 receptor protein is

expressed in the dendritic region and axon terminals of PK cells. It also indicates that the morphology of the PK cells and the density of CB_1 receptors was altered due to alcohol exposure. We expect that these alterations are mediated by disruption of the expression of cnr1a receptor (CB_1) in the brain.

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Introduction

Chronic alcoholism, or alcohol use disorder (AUD), has obvious effects on one's motor skills, such as slurred speech and difficulty walking in a straight line. According to the National Institute on Alcohol Abuse and Alcoholism, 17 million adults were diagnosed with AUD in the United States in 2012, and the criteria include time spent consuming alcohol, amount consumed on average at one time, cravings and influence on normal activities. The effects of chronic alcohol use on the body include stroke, hypertension, cardiomyopathy, cirrhosis of the liver and various forms of cancer. Clearly, this is a wide-ranging problem that crosses socioeconomic boundaries and has varied effects on an individual.

The exact molecular mechanism of alcohol's effects on the brain is the subject of many studies, including one from the University of Bonn in Germany entitled "A Critical Role for the Cannabinoid CB_1 Receptors in Alcohol Dependence and Stress-Stimulated Ethanol Drinking". In this study, Ildiko Racz, et al. (2003) proposed that a target for alcohol in the brain may be the CB_1 receptors of the endocannabinoid system (ECS). The ECS is comprised of cannabinoid receptors, such as CB_1, that are involved in the biosynthesis and degradation of ligands and putative membrane transport proteins (Erdozain and Callado, 2011). Their reasoning includes the similarity in the physiological and behavioral effects of ethanol to those of tetrahydrocannabinol (THC), "the natural CB_1 agonist found in *Cannabis sativa*"

(Racz, et al. 2003). In addition, it has been shown that developmental expression of CB_1 mRNA in Japanese medaka embryos was disrupted by ethanol exposure (Dasmahapatra and Khan, 2013).

The primary focus of this experiment is to investigate the effects of alcohol exposure on the expression of the endocannabinoid receptor 1 (*cnr1a*) gene in adult Japanese medaka (*Oryzias latipes*). CB_1 receptors are produced by this gene (Dasmahaptra and Khan, 2013) and are located on the Purkinje cells, or neuronal cell bodies, in the cerebellum as well as other areas of the medaka brain and are also present in the human central nervous system. The function of CB_1 receptors is not well known, but they are currently being studied for their role in the nervous system due to their abundance in the brain. According to researchers from Brown University and the University of Washington, "the important role of this system in the brain is apparent from the extremely high levels of cannabinoid receptors in the brain… and the profound effects of cannabinoids on motor function, pain sensitivity, cognition, and signaling pathways…" (Tsou, et al., 1997)

Certain stains can bind to the proteins in CB_1 receptors and provide a picture of the amount present. The first part of this study focuses on the distribution of CB_1 receptors in the brain of the Japanese medaka. Japanese medaka were used as a model organism due to their relatively small size as adults, the high number of eggs laid by the female at one time, and the ease with which the sexes can be determined.

Also, the *cnr1a* gene of medaka is an ortholog to that of humans (Figure 1). Genes encoding orthologs of the mammalian *cnr1a* are found throughout the vertebrates, including chickens, turtles, frogs and fish. This indicates the presence of brain cannabinoid receptors in all vertebrate classes (Elphick and Ergetova, 2001).

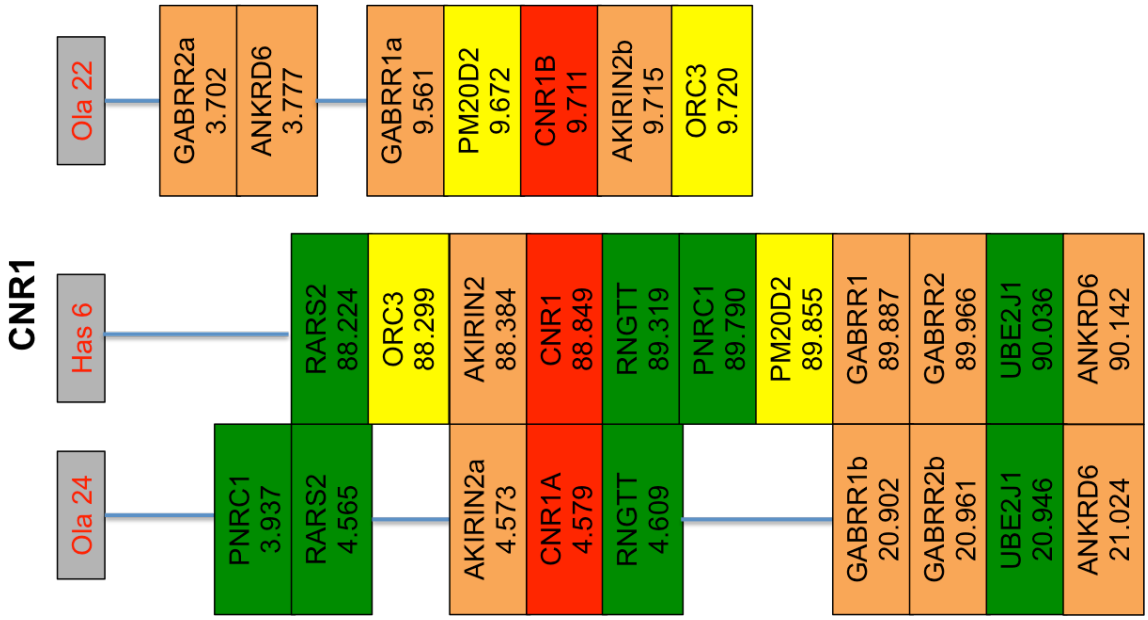

Figure 1: Synteny of medaka cnr1a and human cnr1 show that the two genes, shown in red, are orthologous (Dasmahapatra and Khan, 2013)

Expression of the *cnr1a* gene can be determined by in situ hybridization or immunohistochemical staining and determining the number of CB_1 receptors present. According to Tsou, et al., "the lack of knowledge of the cellular distribution of cannabinoid receptors in the brain represents a significant gap in the literature and has hampered attempts to elucidate the functions of endogenous cannabinoids." Immunohistochemical techniques were used to show the distribution of CB_1 receptors in the whole brain of medaka. The second part dealt with the change in size and appearance of Purkinje cells. Because CB_1 receptors are found on Purkinje cell bodies, the size and abundance of Purkinje cells can be used as an analog for the amount of CB_1 receptors present in the brain. Cresyl violet stain was used to show Purkinje cell morphology in the medaka brain. It was hypothesized that CB_1 receptors would be present in the greatest amount in the cerebellar region of the brain, and that the expression of the *cnr1a* gene would be affected by exposure to alcohol in the brain of the adult Japanese medaka.

Materials and Methods

The Institutional Animal Care and Use Committee (IACUC) of the University of Mississippi approved all experimental protocols.

Fish maintenance

Japanese medaka were kept in freshwater tanks at 25-26°C. Prior to alcohol exposure, the selected fish were removed from their tanks and weighed to determine their age and sex. Secondary sex characteristics were examined under a light microscope. Adult females hold their eggs in their anal tail, and males do not. Only adult males were used for the present study.

Localization of CB_1 receptors in medaka brain using immunochistochemistry

Tissue collection and processing: To determine the distribution of CB_1 receptors in the brain of the Japanese medaka, a group of 12 adult male medaka were selected for study. They were placed in hatching solution at 18°C in separate containers. They were each sedated using MS-222 (tricaine methanesulfonate, 500 mg/1000 mL hatching solution), and their heads were removed and placed in 4% paraformaldehyde solution for 24 hours. Then, the sections were washed with nanopure water and subjected to an alcohol series for dehydration. They were placed in 10 mL of 30% ethanol solution for 2 hours, 50% for 2 hours, 70% for 24

hours, 90% for 6 hours and 100% ethanol solution for 24 hours. Finally, they were placed in 10 mL xylene solution for 2 hours for clearing, after which they were placed in paraffin for 22 hours at 60°C. They were then poured into individual block molds along with the melted paraffin and stored overnight in a freezer.

After freezing, the medaka tissues were then cut into 4μm sections using a microtome and placed on microscope slides. Approximately 12 sections were cut in series from each medaka. They were then deparaffinized by washing in xylene for 15 minutes and rehydrated by washing in solutions of 100%, 95% and 80% alcohol for 3 minutes each. Then, the slides were stained using an immunohistochemistry protocol (IHC-plus™, LifeSpan BioSciences, Inc.).

Immunostaining: In the antigen retrieval step of the procedure, the slides were steamed in a sodium citrate buffer (0.1M, pH 6.0) at 100°C for 20 minutes, then allowed to stand at room temperature for 20 minutes. Then they were rinsed in 1x phosphate-buffered saline solution with Tween (PBST). In the immunostaining step, a PBST and albumin solution (10μL per 1mL) was used as a protein blocker (1% BSA) and applied to the slides for 20 minutes. Then, the primary antibody (IHC-Plus) was applied to the slides, followed by rinsing with PBST. The biotinylated secondary antibody was then applied, and again the slides were washed with PBST. After both antibodies were applied to the specimens, they were stained using alkaline phosphatase streptavidin and a phosphatase chromogen substrate. Finally, they were dehydrated by washing in 80%, 95% and 100% alcohol, followed by xylene. The slides were then covered and observed under a light microscope for the

presence of antibodies associated with CB1 receptors in the whole brain region of the medaka.

Treatment of medaka brain with ethanol

Tissue collection and processing: The second part of the experiment studied the effect on size and shape of Purkinje cells in the cerebellum after alcohol exposure. 12 adult male Japanese medaka (*Oryzias latipes*) were placed in hatching solution for one hour at 25°C. Medaka 1-4, the control group, remained in the hatching solution, while the fish numbered 5-12 were placed in an ethanol solution (7 mL EtOH/400 mL hatching solution) for one hour. After an hour, numbers 9-12 were replaced in the pure hatching solution for one hour for recovery. Then, all 12 medaka were sedated using MS-222 (tricaine methanesulfonate, 500 mg/1000 mL hatching solution), and their heads were removed and placed in 4% paraformaldehyde solution for 24 hours. Then, the tissues were washed with nanopure water and subjected to an alcohol series in order to dehydrate them. They were placed in 10 mL of 30% ethanol solution for 2 hours, 50% for 2 hours, 70% for 24 hours, 90% for 6 hours, 100% overnight, and finally xylene for 2 hours to clear the tissues. After clearing, they were placed in paraffin at 60°C for 22 hours, then poured into a block mold and stored overnight in a freezer.

Staining: Once the blocks had frozen, sections were cut using a microtome at 7 μm and placed on microscope slides. Approximately 12 sections were cut from each fish sample. Half of each of the sections were stained using a cresyl violet stain. The sections were first placed in xylene for 5 minutes, then a series of decreasing alcohol concentrations: 100%, 90%, 70%, 50% and 30%, each for 5 minutes. They

were then washed with water and stained with a 0.5% cresyl violet stain (500mg crystal violet, 25 mL methanol, 75 mL water, 4-5 drops of acetic acid) for 2 minutes. The slides were then rinsed with water and subjected to an increasing alcohol series, from 30% to 90%, followed by 1 minute in eosin dye, an hour in 100% ethanol and 15 minutes in xylene. The slides were then mounted using paramount and examined under a microscope to determine the size and number of Purkinje cells located in the medaka cerebellum.

Scanning electron microscopy

After sections had been cut from the paraffin blocks of control medaka, the remaining tissue was transferred to xylene for removal of paraffin. The sections were left overnight in 100% ethanol. They were then critical point-dried using a critical point drier (Denton Vacuum, Moorestown, NJ, USA). Mid-sagittal sections of were photographed using a JSM-5600 scanning electron microscope (JEOL Ltd., Tokyo, Japan) to give pictures of the whole brain, the whole cerebellum and the three distinct layers of the cerebellum.

Observations and Results

Medaka cerebellum

Based on information gathered from the following micrograph pictures, the medaka cerebellum appears to be similar to the human cerebellum in that there are three distinct layers: the granular layer, Purkinje layer and molecular layer. A 2009 study of zebrafish, a close relative of medaka, showed that there are three major regions of their cerebellum: the valvula cerebellum, the corpus cerebellum and the cristae cerebellum. The valvula and corpus cerebelli contain the three-layer structure characteristic of human cerebelli, but the cristae region only contains the granular layer. This study also stated that there are few significant differences between the zebrafish and human cerebellum (Bae, et al., 2009).

Localization of CB_1 receptors in the medaka brain

Based on cresyl violet-stained sections and scanning electron micrographs (SEM) of the whole medaka brain, the location of the cerebellar region was fairly easy to locate and from there, the three distinct layers of the cerebellum were determined. The granular layer contained the greatest abundance of violet staining granules, the Purkinje layer contained the larger, lighter staining neuronal cell bodies, and the molecular layer had virtually no granules present. The present study

focused largely on the region of the cerebellum known as the corpus cerebellum, or the body.

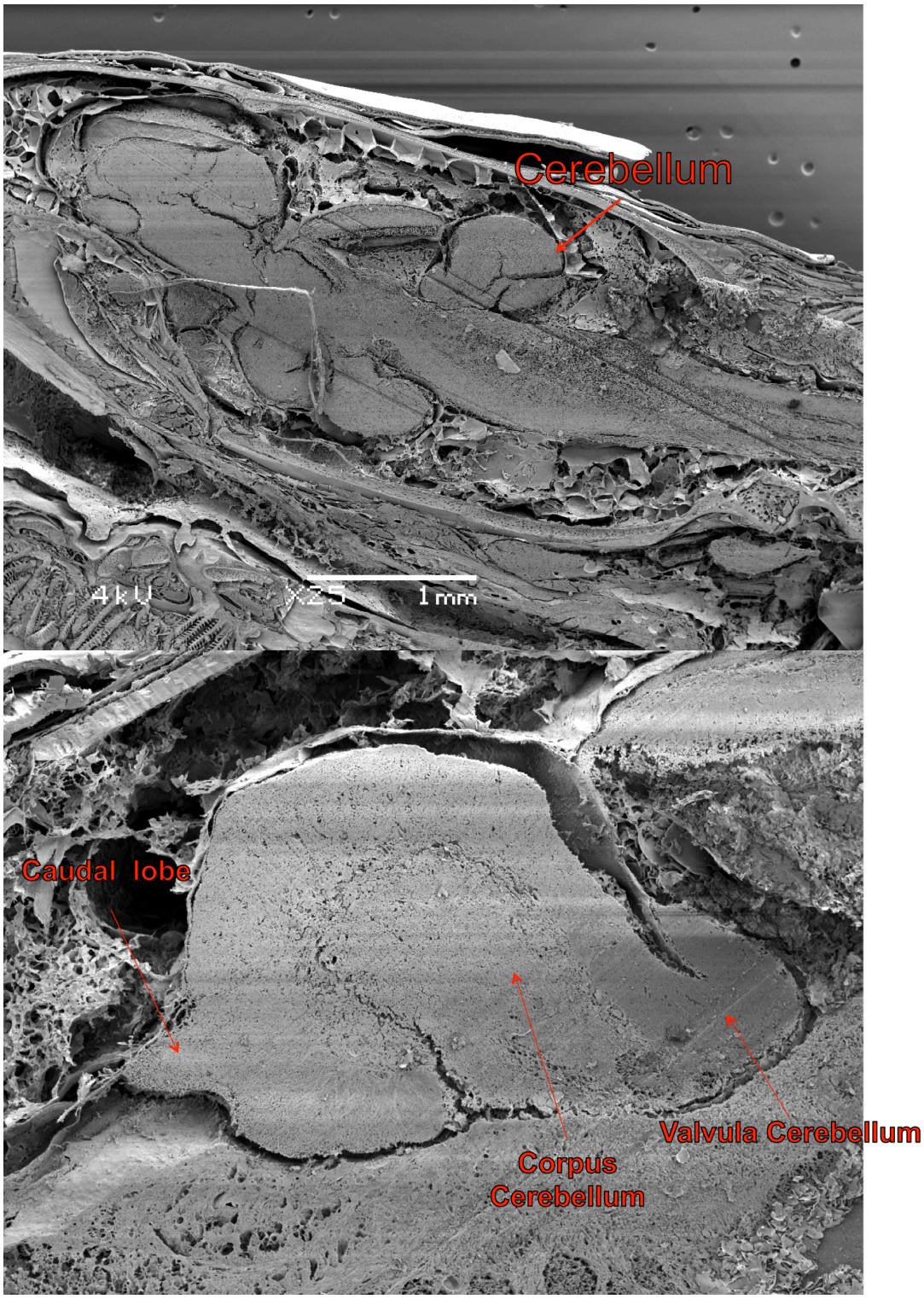

Figure 2: SEM micrographs of untreated medaka whole brain, showing the cerebellum (top) as well as the three distinct regions of the cerebellum (bottom)

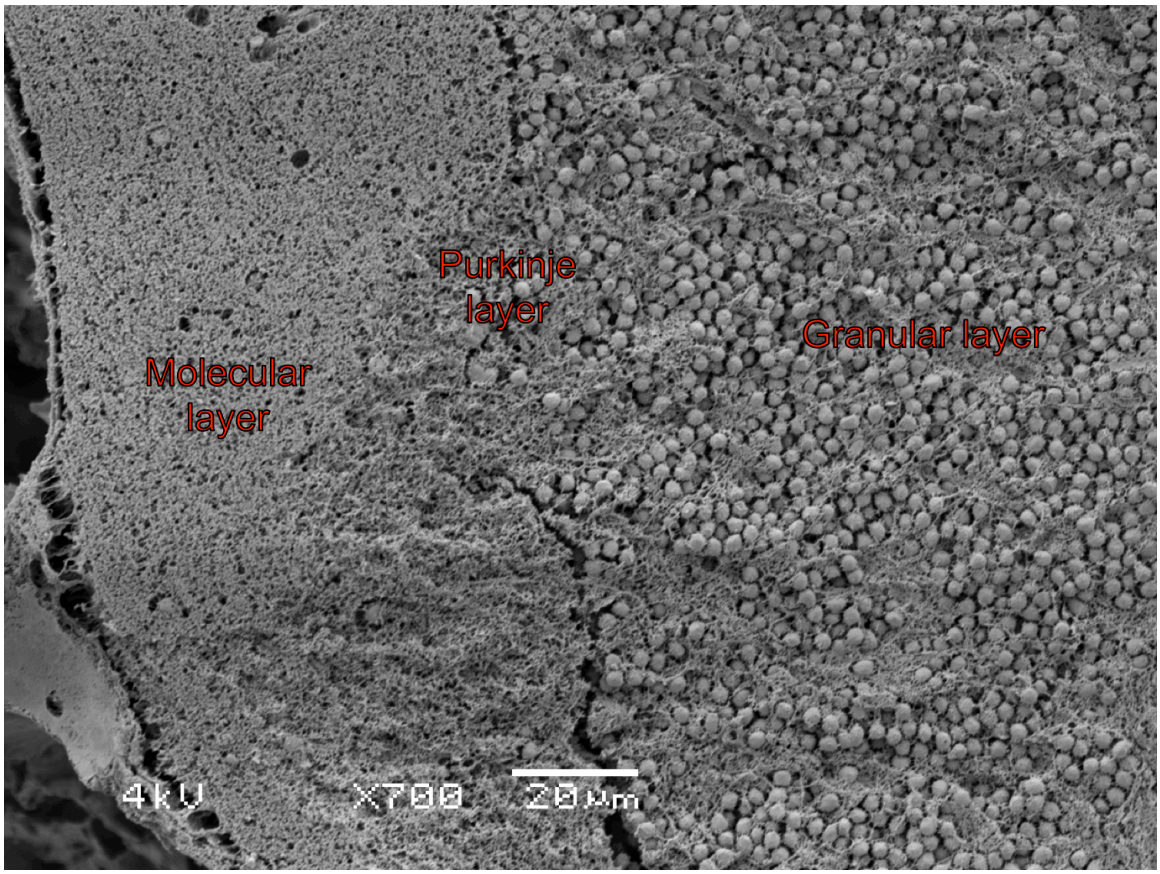

Figure 3: SEM micrograph showing the three distinct layers of the cerebellum. The large Purkinje cell bodies are visible on the border between the molecular and granular layers.

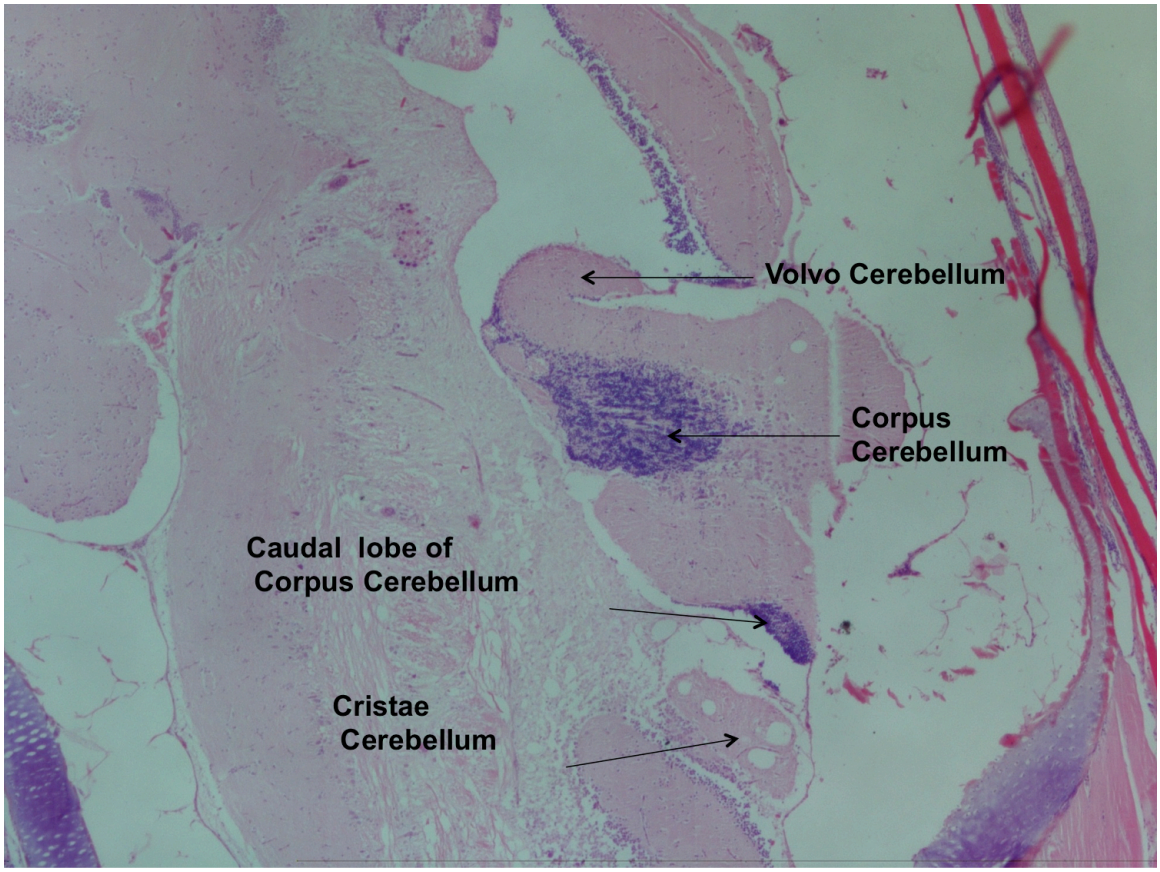

Figure 4: Light microscopic view of cresyl violet stained medaka whole brain shows the three regions of the cerebellum, as well as the darkly staining areas of the granular layer (4x)

The immunohistochemical (IHC) staining used in this experiment was designed to react with antibodies associated with CB_1 receptor proteins and give an accurate picture of where they occur with the highest density in the medaka brain. In this part of the study, the effects of alcohol on the distribution of receptors was not considered. The medaka used ranged in weight from 0.250mg to 0.580mg, indicating that they were all adults. Their sexes were determined by examining their anal fins for the presence of papillae. They were all determined to be male. The IHC-stained sections showed that the area of the cerebellum with the highest abundance of CB_1 receptors occurred in the granular layer of the corpus cerebellum. There was also immunoreactivity present in the Purkinje layer on the surface of the neuronal cell bodies, possibly on the dendrites. The molecular layer showed virtually no CB_1 receptor immunoreactivity.

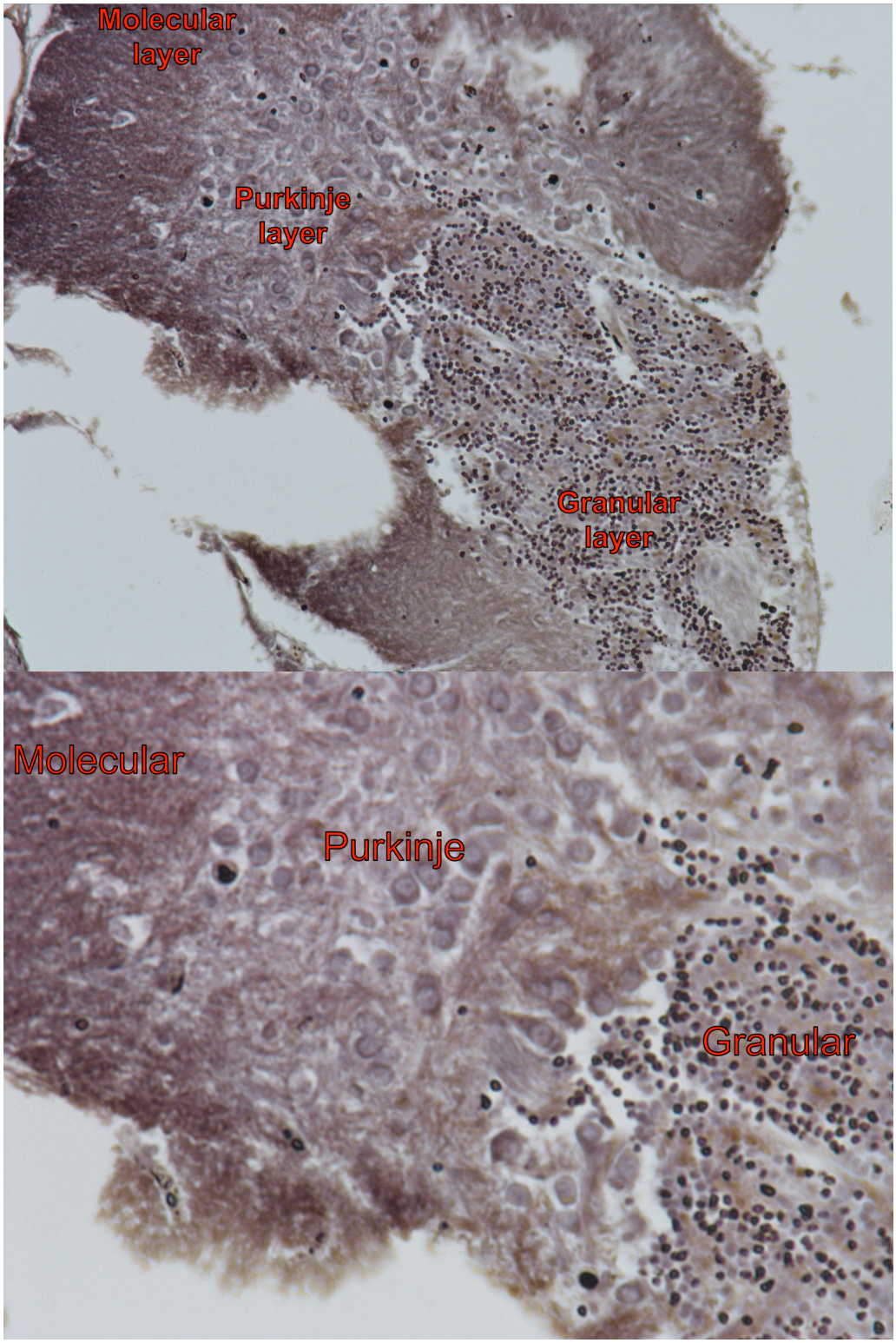

Figure 5: Light microscopic view of immunostained medaka cerebellum showing highest density of immunoreactivity in the granular layer, with some in Purkinje layer (top 10x, bottom 40x)

Effects of ethanol exposure on Purkinje cells and CB_1 receptors

From the beginning of the alcohol treatment portion of the study, it was obvious that the treated fish were behaviorally and physiologically affected. Within one minute of exposure to the ethanol solution, they turned on their sides and became immobile due to loss of motor function.

Figure 6: Medaka being treated with ethanol. Four of the twelve were replaced in their standard hatching solution after an hour in alcohol, allowing for recovery.

Figure 7: After about an hour of alcohol exposure, the medaka lost motor function and swimming ability.

However, fish 9-12 regained motility and function after being replaced in the hatching solution. The fish ranged in weight from 0.270mg to 0.595mg, indicating they were all adults.

Examination of the stained tissue sections using a compound microscope indicates a couple of general trends in the number of CB_1 receptors present and the size of the Purkinje cells in the cerebellum region of the brain. Based solely on observational data, there appeared to be more receptors present in the control group (Figures 8, 9) than in the alcohol plus recovery group (Fig. 10), which in turn had more receptors than the group that was not allowed to recover (Fig. 11). Also, the size and shape of the Purkinje cells changed with different alcohol exposures.

The control group's Purkinje cells were smaller and more clearly defined (Fig. 8, 9); whereas the recovery group had slightly larger and less defined Purkinje cells (Fig. 10). The non-recovery group had the largest Purkinje cells, and they were almost "fuzzy" in appearance (Fig. 11).

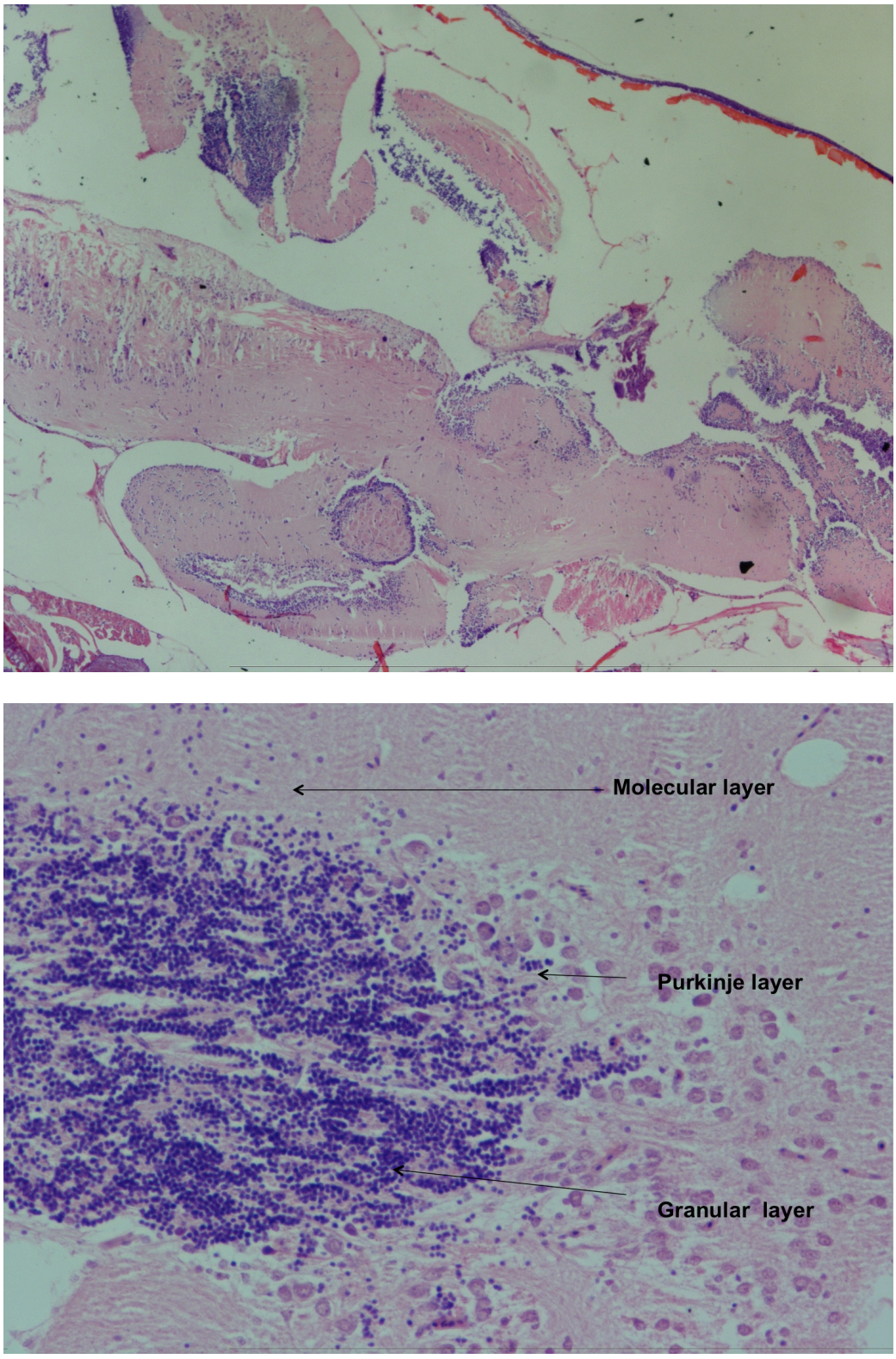

Figure 8: Light microscopic view of a control medaka whole brain (top 4x) and cerebellum (bottom 10x), showing three layers of cerebellum, the darkly staining CB_1 receptors in the granular layer and the larger PK cells in the Purkinje layer.

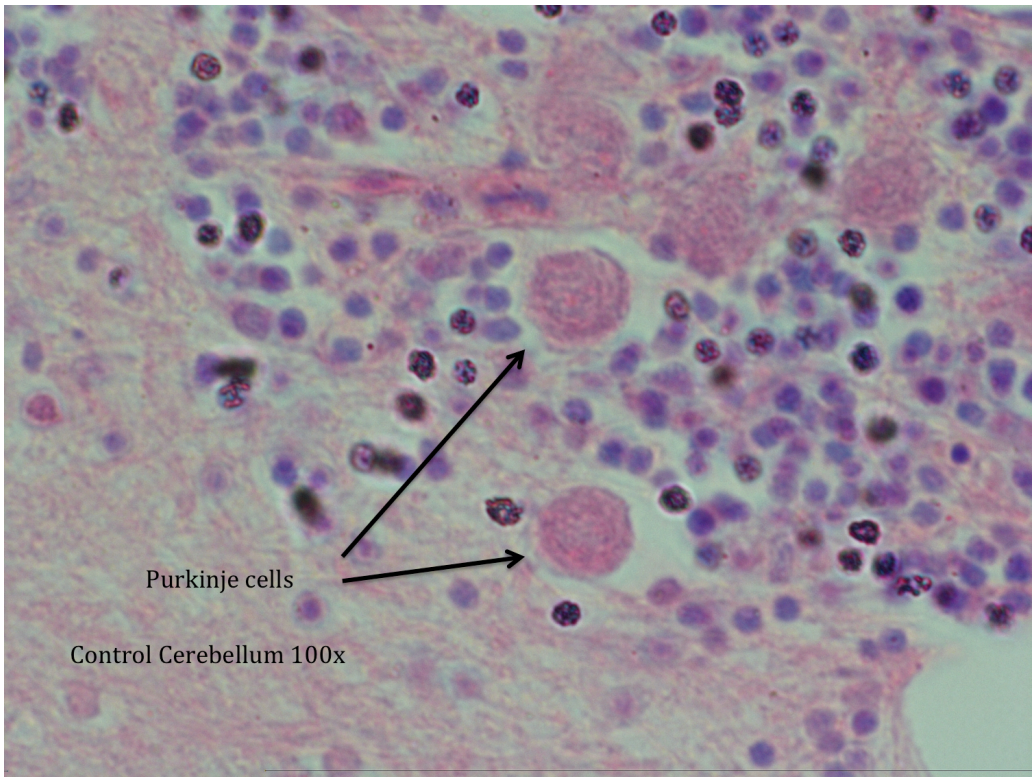

Figure 9: Light microscopic view of control medaka cerebellum Purkinje and granular layers (100x)

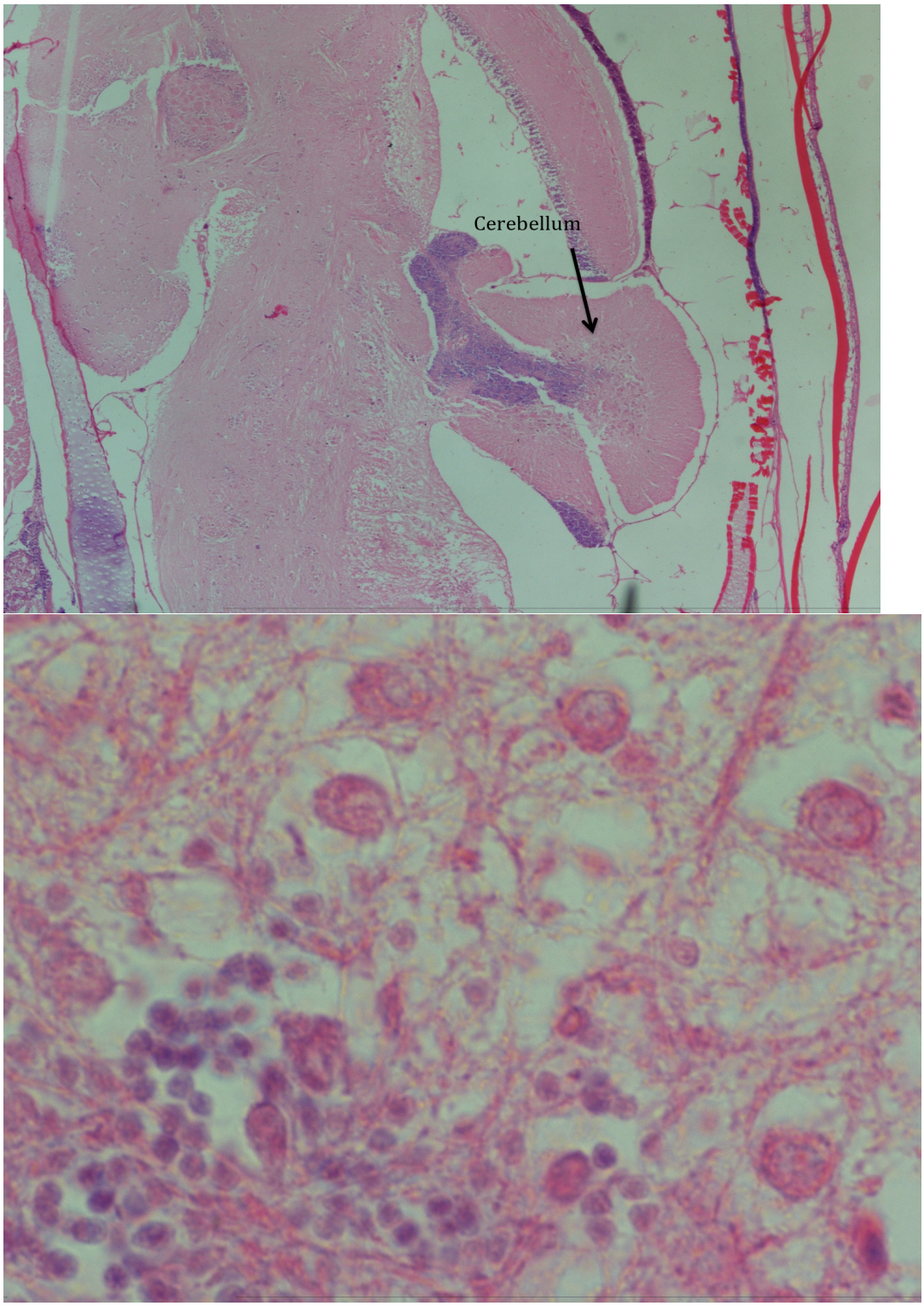

Figure 10: Light microscopic view of medaka cerebellum that was allowed to recover for 1 hour. Top (cresyl violet stain, 4x) shows lower density of CB_1 receptors in granular layer. Bottom (cresyl violet stain, 100x) shows slightly larger PK cells in Purkinje layer.

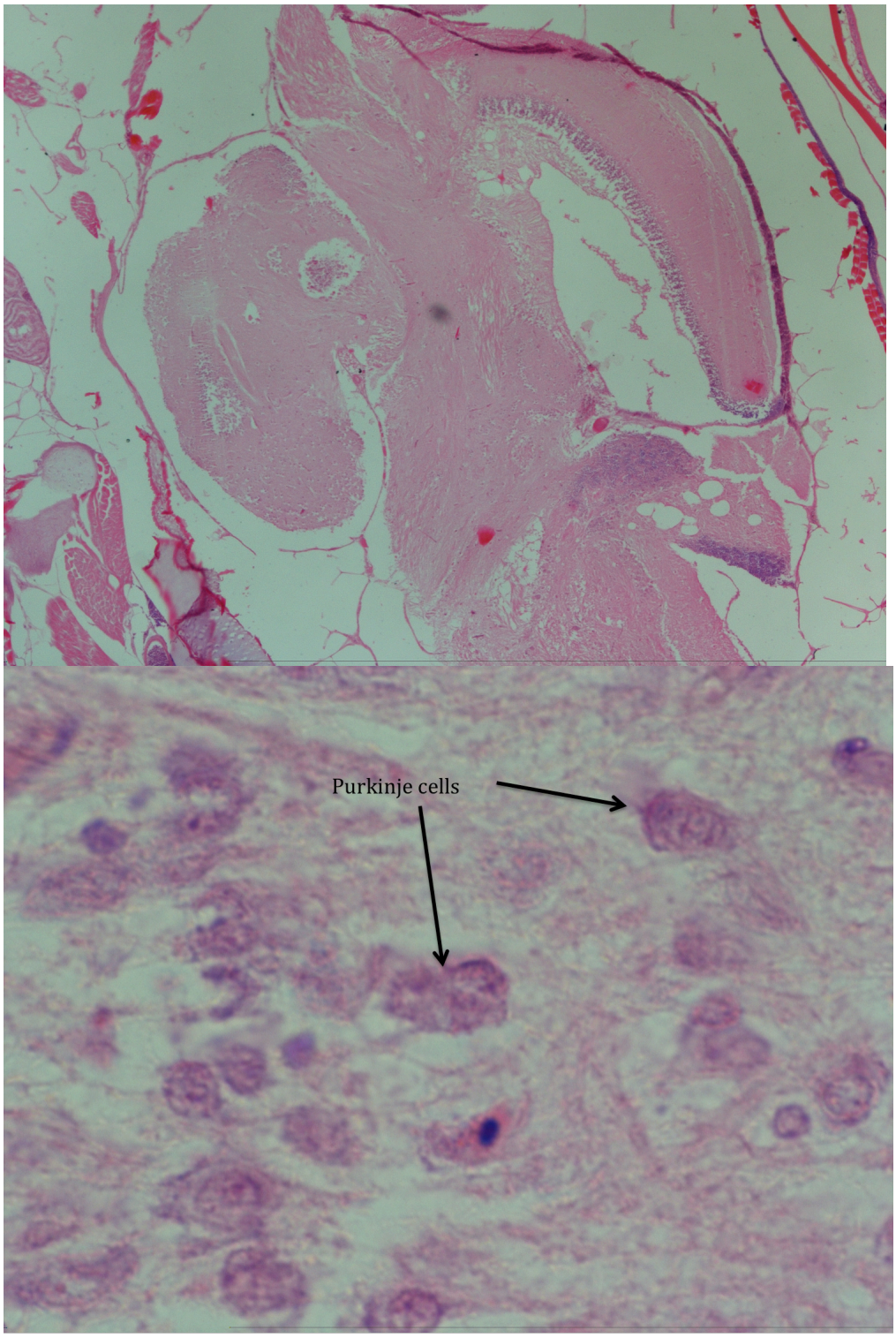

Figure 11: Light microscopic views of medaka cerebellum that had been treated with alcohol and not allowed to recover. 4x (top, cresyl violet stain) and 100x (bottom, cresyl violet) views show the lowest density of receptors and hypertrophied PK cells.

Discussion

The present study was focused on the CB_1 endocannabinoid receptors of Japanese rice fish as a potential target of ethanol for inducing AUD behavior. In order to better understand the relationship between human and medaka cnr1 genes, a gene synteny (Fig. 1) was used to show that the two are paralogous to each other (Dasmahapatra and Khan, 2013). We have gathered information indicating that the CB_1 receptor may be a potential target for ethanol in development and behavior. We have then used immunohistochemical staining to determine the distribution of CB_1 receptors in the medaka brain, and finally we have used alcohol exposure and cresyl violet staining to determine the effects of ethanol on the morphology of Purkinje cells and the expression of the *cnr1a* gene in medaka.

Our data indicate that the highest density of CB_1 receptors in the brain occur in the granular layer of the cerebellum, the region of the brain tasked with fine motor control and balance. There was also some immunoreactivity in the Purkinje layer. It is probable that some CB_1 receptors are located on the dendritic region of the cell body, in the Purkinje layer, and the majority is located on the axon terminals, in the granular layer. If CB_1 receptors are indeed a target for chronic alcohol exposure in adults, it can be assumed that behavioral effects would be seen in the form of loss of balance and motor function, which has been observed to be true. Once the medaka were exposed to ethanol for up to an hour, they turned over

and were rendered immobile. The exposed fish also experienced a reduction in the number of CB_1 receptors in the cerebellum, as well as hypertrophy of the Purkinje cells.

From this data, we hope that the mechanism of ethanol exposure on the brain on a molecular level may be better understood. The ultimate goal of this ongoing study is to determine a method for attenuating the effects of prolonged alcohol exposure on adults suffering from alcohol use disorders. It was shown that once the medaka were returned to the hatching solution after prolonged alcohol exposure, they regained motor function and resumed normal swimming behavior. Moreover, examination of the receptor density and Purkinje cell morphology indicated that each had returned to levels close to the control group. While this indicates that the effects may be attenuated by removing ethanol exposure, this is a reactive approach and does not address the longer term consequences. In the future, Dr. Asok Dasmahapatra will examine the ability of natural products to lessen the effects of ethanol exposure *in vivo* as a more proactive method and effective treatment.

In summary, the results of this study indicate that exposure to alcohol does affect the expression of CB_1 receptors in the cerebellum of Japanese medaka. The effects are shown in the loss of motor function during alcohol exposure of the live fish as well as a reduction in the number of receptors present in the cerebellum during examination of the brain tissue. Also, the size and shape of the Purkinje cells are altered when the brain is exposed to alcohol.

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